Saskatchewan
Agriculture, Food
and Rural
Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

19930025

DEVELOPMENT AND ASSESSMENT OF NEW ADJUVANTS FOR BETTER VACCINES

Funded by: The Agriculture Development Fund

August, 1997

Prepared by: Vaccine & Infectious Disease Organization

Final Report

DEVELOPMENT AND ASSESSMENT OF NEW ADJUVANTS FOR BETTER VACCINES

93000025

Funded by
The Agriculture Development Fund
August 1997

Prepared by: VIDO

The views expressed in this report are those of the author(s).
The Agriculture Development Fund (ADF) does not accept responsibility or liability for any conclusions or recommendations in the report, and cannot guarantee the accuracy of its data or observations.

105-07384/

ADF FINAL REPORT

#953000025 - "Development and Assessment of New Adjuvants for Better Vaccines"

by

Maria Baca-Estrada
Veterinary Infectious Disease Organization
120 Veterinary Road
Saskatoon, SK Canada S7N 5E3

SUMMARY

Vaccination can be effective method of disease prevention by stimulating the naive animal to develop protective immunity against the disease-causing organism. However, in order to optimize the response, inactivated vaccines need to be formulated with a component called an adjuvant. Only a few adjuvants are licensed for veterinary use because most adjuvants induce strong tissue reactions or create residue problems which can affect the quality of the carcass. An effective and safe adjuvant is a critical component for the new generation of killed and recombinant protein vaccines.

We have examined the ability of liposomes as vaccine delivery systems. In addition to their safety, liposomes have been shown to be able to enhanced the immune response to vaccines in may animal species. As outlined in the previous report, our objective was to evaluate the immune response to different liposome formulations in mice. In summary, we have successfully completed many of the milestones outlined in the original application and continue to develop strategies to improve vaccine adjuvants and immunization methods that will be of benefit to the livestock industry.

TECHNICAL REPORT

A) BACKGROUND AND OBJECTIVES

Inactivated vaccines contain two critical components: the antigen and the adjuvant. The antigen, either a part of or the entire disease causing organism, is responsible for inducing the specific immunity which protects the animal against the disease. The adjuvant is important because it enhances the immune response to the antigen and thus increases the effectiveness of the vaccine. However, currently licensed adjuvants do not stimulate strong immune responses when compared to experimental adjuvants and they also cause

excessive tissue damage that can result in trimming of the carcass. Furthermore, some of the adjuvants now in use will not be licensed for use in the future because of tissue residue problems (eg. non-metabolizable mineral oils).

The main objective of this proposal is to develop and assess new adjuvant formulations that are affordable, safe and effective for use in animal vaccines. Our evaluation of new adjuvants is now being done with protective antigen from the BHV-1 virus called gD and antigens from *Actinobacillus pleuroneumoniae*. We are also starting to assess other antigens from *P. haemolytica (Tbp1 and Tbp2)*. In addition, we have developed assays to measure different immune responses to these antigens. These responses contribute to protection from disease and include assays for both humoral and cell-mediated immunity.

B) PROJECT ACTIVITIES, ACHIEVEMENTS, PUBLICATIONS

The results generated from this project have resulted in a number of publications:

Baca-Estrada, M.E. *et al.* Immunogenicity of bovine herpesvirus 1 glycoprotein gD in mice. Effect of antigen form in the induction of cellular and humoral immune responses. Viral Immunology. 1996. 9: 11-22.

Baca-Estrada, M.E. *et al.* Effect of IL-4 and IL-12 liposomal formulations on the induction of immune responses to bovine herpesvirus type-1 glycoprotein D. Vaccine. in press.

Our main focus during the last two years of this project has been on the development of liposomes as vaccine delivery systems. We have established a collaboration with Dr. M. Foldvari from the College of Pharmacy, U of S.

The results of this project have also been presented at a different national and international scientific meetings:

Willson, P.J. Application of molecular biology to the production of new vaccines for swine. Banff Pork Seminar. 1994. Banff, AB.

Babiuk, L. A. Do vaccines work? 3rd Congress of the European society of veterinary virology. 1994. Switzerland.

Harland, R.J. Immunizing against bovine respiratory disease. Formation continue, corporation professionnelle des medicine veterinaries du Quebec. 1994. Montreal.

Yarosh, O. *et al.* Effect of alum and VSA on the humoral and cellular immune response to BHV-1 gD. Canadian society for immunology, 1995. Lake Louise, AB.

Baca-Estrada, M.E. *et al.* The effect of antigen form of BHV-1 gD on the induction of cellular and humoral immune responses. Symposium on IBR and other ruminant herpesvirus infection.1996. Liege, Belgium.

Willson, P.J. *Streptococcus suis*: Molecular immunity and vaccine develcpment. Presentation to SPI marketing group research committee. 1996. Saskatoon.

Baca-Estrada, M.E. *et al.* Immunogenicity of bovine herpesvirus 1 glycoprotein D in mice: Effect of antigen form on the induction of cellular and humoral immune respones. 9th International congress of immunology, 1995.San Francisco, California.

Baca-Estrada, M.E. *et al.* Effect of IL-4 and IL-12 liposomal formulations on the induction of mucosal and systemic immune responses to bovine herpesvirus type-1 glycoprotein D. 9th International Congress of Mucosal Immunology. 1997, Sydney, Australia. Immunology and Cell Biology 75:A128.

Babiuk, L.A. *et al.* Revolutionizing vaccination through biotechnology. 9th International Congress of Mucosal Immunology. 1997. Sydney, Australia. Immunology and Cell Biology 75:A8, 1997.

C) RESULTS (According to Milestones Outlined in the Original Application) AND CONCLUSIONS

Milestone 1. Modification of antigen and assessment in mice.

Work at VIDO has identified an antigen (gD) from BHV-1 that can induce protective immunity in immunized cattle. However, for the manufacture of veterinary subunit vaccines, modifications to the antigen are needed to make the production of these vaccines cost effective. Recently, economical methods to produce gD have been developed at VIDO which produces two different recombinant gD molecules (glycosylated and non-glycosylated). In order to evaluate whether the modifications made to these proteins did not affect their ability to induce immune responses, mice were vaccinated with the three types of gD antigens (authentic, recombinant glycosylated and recombinant non-glycosylated) and the immune response determined by ELISA and blastogenesis assays.

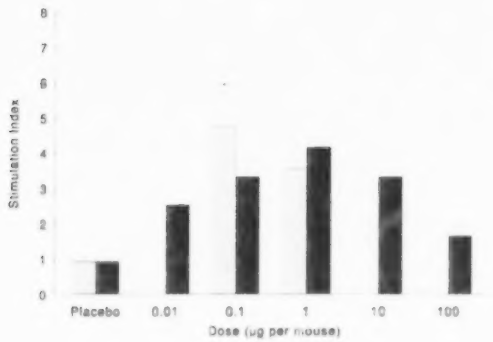
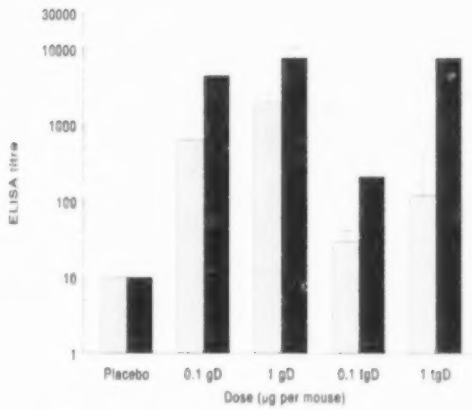


Fig.1. Cellular immune responses to gD following vaccination with different amounts of authentic (gD, empty bars) or truncated (tgD, solid bars) antigens. No statistical differences were observed between the groups. Results are expressed as the mean stimulation index of 5 mice + standard deviation.

Fig 2. Antibody responses to gD following one (empty bars) or two vaccinations (solid bar) with different amounts of gD or tgD. The results demonstrated that the primary vaccination with tgD induces significantly lower antibody titres than vaccination with gD. However, no statistical differences were observed after a secondary vaccination. The results are expressed as the geometric mean titre of 5 mice + standard deviation.



The experiments in the initial phase of this project have confirmed the importance of antigen form in the induction of immune responses. The results demonstrated that although tgD is less efficient in inducing primary immune responses than the authentic form of the molecule, tgD it is capable of stimulating strong secondary cellular (Fig 1) and antibody (Fig 2) responses.

Milestone 2. Development of oil emulsion modifications study in mice.

We have also carried out experiments to evaluate different mineral oil-based emulsions formulations including VSA, a formulation developed at VIDO. The study was carried out in mice as the activation pathways are somewhat better defined than in cattle. C57BL/6 mice were immunized in the left footpad with 0.5 μg of gD in alum or in VSA as adjuvants, then reimmunized by the same route on day 70. Mice immunized with gD in VSA were able to invoke an earlier humoral response with significantly higher titres of neutralizing antibodies than gD/alum immunized mice (Fig 3).

Significant *in vitro* lymphocyte proliferation to gD was not detected until after reimmunization; splenic cells from mice immunized with gD in VSA demonstrated higher proliferative responses than mice immunized with gD in alum (data not shown).

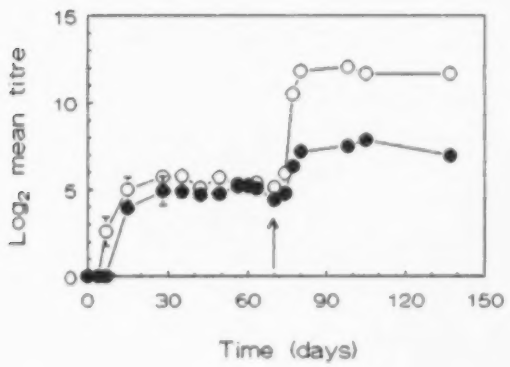


Fig 3. Antibody responses to gD following two vaccinations (day 0 and day 70) with gD in Alum (solid circle) or in VSA (open circle). The results demonstrated that immunization with VSA stimulates a stronger secondary antibody response when compared to that induced by gD in Alum. The results are expressed as the mean $\log_2$ of 5 mice per group.

In conclusion, we have evaluated a promising mimeral oil emulsion based adjuvant. However, mineral oil can induce long-term irritation in the animal and mineral oil adjuvants are banned in the European community. Further work requires the replacement of the mineral oil with a more suitable, biodegradable oil such as liposomes or with chimeric cytokine molecules as potential adjuvants.

Milestone 3. Assessment of liposomes and ISCOMS in mice.

Liposomes are small phospholipid vesicles capable of entrapping and delivering vaccine antigens. Depending on conditions used for production, liposomes can vary in from as well as in size (from 0.01 mm to 150 mm). The two standard types of liposomes are multilamellar and unilamellar. Multilamellar vesicles (MLV) have several lipid bilayers. Unilamellar vesicles have a single bilayer membrane and can be small (SUV) or large (LUV). Liposomes can also vary in their stability, fluidity and permeability depending on the ratio of phospholipid to cholesterol. The surface of liposomes can be neutral, negatively or positively charged. Each of these properties influences how effective a liposome will be as a delivery system for different vaccine antigens. Liposomes are currently used as drug delivery systems in humans, and are presently being tested for vaccine delivery purposes. Therefore, it is anticipated that regulatory barriers will not prevent this technology from being used in the veterinary field.

Our first experiments in this section were designed to assess the BHV-1 gD incorporation efficiency of 12 different liposome formulations. The liposome formulations varied in charge (positive, negative and neutral), structure (MLV and LUV) and in the type of antigen (BHV-1 gD full-length or truncated molecule). Our results demonstrated that different formulations incorporated different amounts of gD: + MLV > - MLV > nMLV (Fig 4). Similar results were observed with LUV (data not shown). Very high incorporation of BHV-1 gD (up to 85%) was observed in +MLV and -MLV.

These preliminary results are encouraging since the production of liposomes can only be cost effective if incorporation of the antigens is high. We are doing experiments to test these liposome formulations in an experimental model to assess their ability to induce both humoral and cellular immune responses to gD.

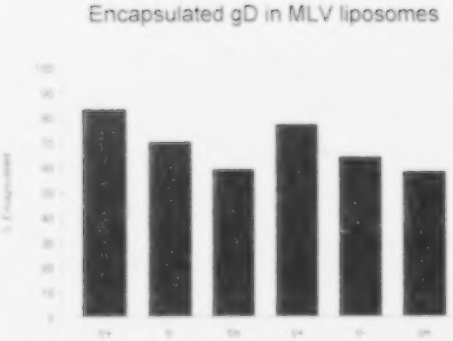


Fig 4. Incorporation efficiency of gD and tgD into multilamellar (MLV) liposomes. The results demonstrated that positive liposomes encapsulated the highest levels of of gD and tgD. Results are expressed as the mean % encapsulation + standard deviation of three separate experiments.

The next step in the characterization of this formulations was to assess their ability to induce immune responses in vivo. The similar encapsulation efficiency of both gD and tgD, together with the fact that the truncated molecule is the antigen use in the field vaccines, we decided to determined the in vivo responses using the tgD antigen formulated in MLV and LUV of different charges (positive, negative and neutral). Our data show that all formulations were capable of inducing antibody responses as measured by ELISA (Fig 5A) However, only the antibodies elicited by the MLV formulations contained antibodies capable of neutralizing BHV-1 infectivity in vitro (Fig 5B), suggesting that the process of extrusion used in the preparation of LUV damages the antigenic structure of the gD antigen. Therefore, the following experiments in this project will be carried out using MLV only.

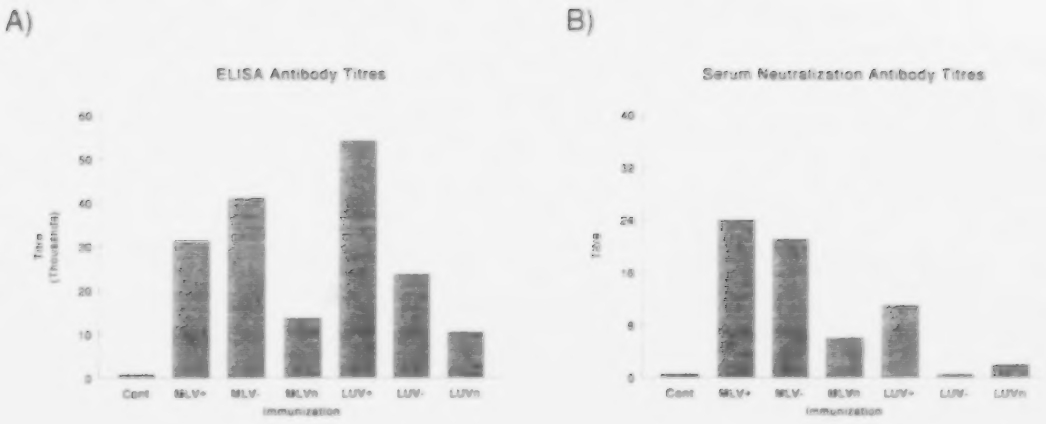


Fig 5. Antibody responses induced by different liposome formulations containing tgD. A) Antibody titers measured by ELISA. B) Serum neutralization titres. These results demonstrated that the composition of the liposomes influences the stimulation of immune responses. Positive and negative MLV were capable of inducing strong ELISA as well as virus neutralization titres when compared to the other liposome formulations.

Stimulation of antigen-specific cell-mediated immune responses is an important feature of an effective vaccine. Therefore, we assessed the ability of these liposome formulations to induce gD-specific cellular responses by measuring proliferative responses (Fig 6) and cytokine secretion (data not shown) in spleen cells of immunized mice.

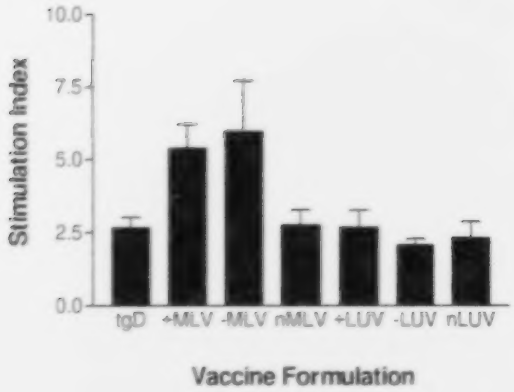


Fig 6. Proliferative responses to gD following immunization with different liposome formulations containing tgD. The results showed that both positive and negative MLV are capable of inducing antigen-specific responses. The data are expressed as the mean stimulation index + standard deviation of 5 mice per group.

We have extended our observations to assess the ability of liposome-gD formulations to induce mucosal immune responses following intranasal immunization.

Our results demonstrated that intranasal immunization of mice with liposomes containing BHV1 gD significantly enhanced the immune responses to gD (Fig 7).

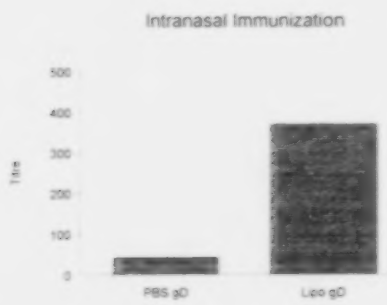
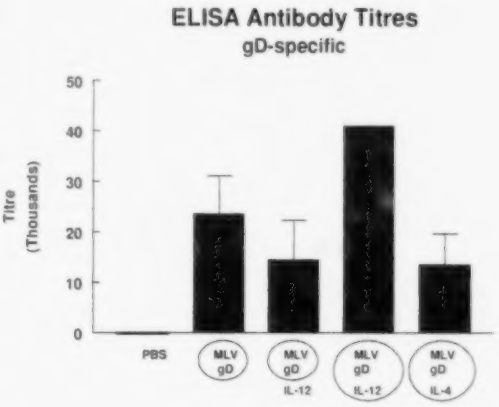


Fig 7. Antibody responses measured by ELISA following intranasal immunization of mice with gD alone or gD incorporated into positive liposomes. The gD liposome formulation was able to induce significantly higher antibody responses than gD in PBS. The results are expressed as the geometric mean titre + standard deviation of 5 mice per group.

One of the objectives of this project was to assess the adjuvant activity of cytokines in this model. We formulated liposomes containing IL-4 and IL-12 since these cytokines have been known to induce activation of different T helper cell activities. T helper 1 (Th1) and T helper 2 (Th2) refer to different types of immune responses. Th1 responses are mediated by IFN-γ and are important to protect the host against most viral and some bacterial diseases. In contrast, Th2

responses are mediated by secretion of cytokines such as IL-4 and are important in protecting against some bacterial diseases.

Fig 8. Antibody responses in serum of immunized mice with different multilamellar (MLV) liposomes containing antigen alone or with either IL-12 or IL-4. These results demonstrated that incorporation of IL-12 into the liposome has a significant effect in the induction of anti-gD antibody responses. The results are expressed as the geometric mean titre + standard deviation of 5 mice per group.



We examined, therefore, the immune responses elicited by mucosal or systemic immunization of mice with liposome formulations containing bovine herpesvirus type-1 glycoprotein D (gD) together with IL-4 or IL-12. Subcutaneous immunization with liposomes containing gD and IL-12 significantly enhanced the induction of antigen-specific cellular (data not shown) and humoral (Fig 8) immune responses.

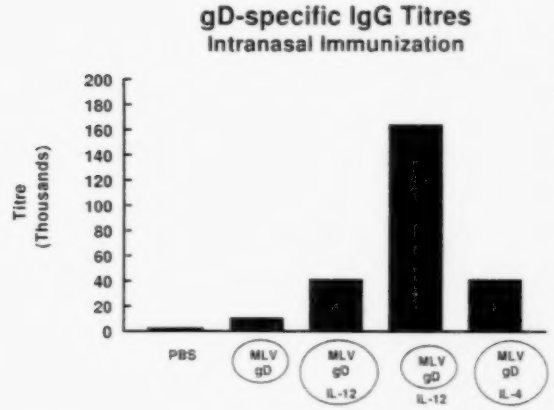


Fig 9. Antibody responses in serum following intranasal immunization of mice with different liposome formulations containing antigen or antigen and cytokine. The results demonstrated that the presence of IL-12 out side the liposome is more efficient in inducing adjuvant effect than the incorporation of this cytokine into the liposome. The results are expressed as the geometric mean titre + standard deviation of 5 mice per group.

These responses were characterized by the increase on IFN-γ secreting cells and the induction of gD-specific IgG2a antibodies (data not shown). In contrast, incorporation of IL-4 into gD-liposomes did not enhanced the humoral immune response to gD but significantly influenced the development of antigen-specific IL-4 secreting cells. Intranasal immunization with IL-12 gD-liposome formulations did not alter the induction of mucosal immune responses when compared to gD-liposomes. However, a significant increase in systemic immune responses were induced in IL-12 gD-liposome immunized mice by the intranasal route (Fig 9). While incorporation of IL-12 into the liposomes was necessary for the systemic adjuvant effect, incorporation of the cytokine was not essential for the enhancement of immune responses when the animals were immunized by the intranasal route (Fig 9). These findings indicate that the requirements for cytokines to influence immune responses to a vaccine antigen differ between systemic and mucosal routes.

Milestone 4. Vaccination with best adjuvant in cattle.
Milestone 6. Serology of vaccinated cattle and challenge with wild BHV-1.

We investigated the ability of different adjuvants to enhance immune responses of cattle to BHV-1 gD with the aim of selecting suitable adjuvants for subunit vaccines against bovine respiratory disease (shipping fever). Since it is anticipated that the adjuvants will function in a similar mode for other subunit antigens in cattle we feel that the gD antigen is a suitable model. Two milestones are reported together since the same animals that were used to evaluate the adjuvants were used to test protection against BHV-1 infection. Table 1 summarizes the efficacy of different vaccines formulations (adjuvants) in inducing both ELISA and virus neutralizing antibodies.

Table 1. Summary table of adjuvants used in the different trials and their effectiveness in inducing immune responses in cattle.

Adjuvant	Characteristics	Adjuvant Effect Found in Cattle
Avridine	Chemically manufactured	Effective
FIA	Derived from bacteria	Effective
QuilA	Derived from a plant	Less effective
Emulsigen	Proprietory formulation	Less effective
Alhydrogel	Aluminum salt	Average
MDP	Derived from bacteria	Average
DDA	Chemically manufactured	Average

The results of this study indicated that immunization of animals with gD and Avridine, FIA, Emulsigen and to a lesser extent QuilA and DDA was able to induce a response significantly higher than immunization in the absence of an adjuvant (Table 3). Although FIA is considered to be the Gold Standard, it cannot be used in animals since it contains a non-metabolized mineral oil. Since Avridine was as effective as FIA it suggested that this adjuvant could be used in vaccine formulations for cattle.

Table 2. Comparison of mean antibody titres induced by different adjuvants assessed by serum neutralization and ELISA ten days following second immunization (animals immunized at day 0 and day 21). Trial #1.

Vaccine Formulation	Serum Neutralization[1]	ELISA[2]
Placebo (none)	32	0.64
gD Avridine	3072	102.4
gD FIA	3072	102.4
gD QuilA	768	25.6
gD DDA	ND	25.6
gD MDP	ND	25.6

[1] mean titre

[2] mean titre x 1000

ND = not done

Once we showed that Avridine was as effective as FIA we then extended these studies to incorporate other adjuvants using Avridine as the standard. Table 3 compares the efficacy of 5 vaccine formulations containing gD or tgD.

Table 3. Comparison of mean antibody titres induced by different adjuvants assessed by serum neutralization and ELISA ten days following second immunization (animals immunized at day 0 and day 21). Trial #2.

Vaccine Formulation	Serum Neutralization[1]	ELISA[2]
Placebo (Avridine)	11±10	0.02
gD Avridine	1045±159	450
tgD Avridine	385±71	49
tgD Emulsigen	298±154	5.1
tgD VSA	757±185	73
tgD Carbopol	72±16	5.1

[1] mean ± SEM

[2] mean anti-gD titre x 1000

Animals immunized with Avridine and VSA tended to have the highest titres following immunization, while those immunized with the other adjuvants had significantly lower titres (Table 3).

Following challenge, nasal swabs were taken from selected groups to assess how much virus was shed, and how this may be related to antibody titres immediately prior to challenge. Those animals that received the adjuvants Avridine or VSA had the least amount of virus shedding, when compared to placebo. However, these data should be regarded as being trends only, as the differences between groups were not significant.

Mucosal immunization of cattle using a liposome based vaccine.

An experiment was designed to assess the ability of gD-liposome formulations to induce mucosal immunity in cattle. Groups of 10 animals were immunized intranasally with formulations containing placebo, gD alone (25 μg/animal) and gD in liposomes (25 μg/animal). The results of this experiment are shown in Fig. 9 and 10.

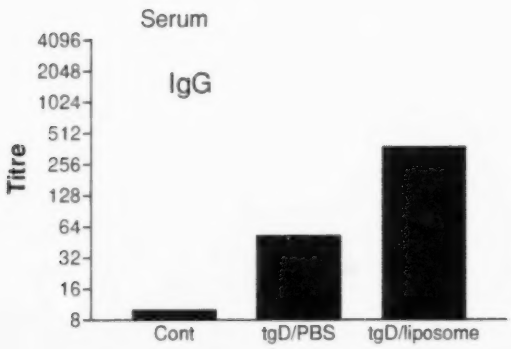
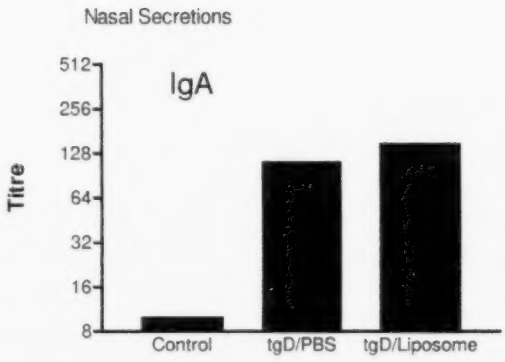


Fig. 9. Antibody response to gD in serum of immunized cattle. Animals were immunized intrnasally and boosted 21 days later. Serum samples were collected 10 days after the second immunization.

Fig. 10. Antibody responses in the mucosal secretions following intranasal immunization. Animals were immunized as in Fig.9. The gD-specific IgA titres were measured in nasal secretions.



Intranasal vaccination with liposome formulations induced significantly higher gD-specific IgG responses in serum. Interestingly, the gD-specific IgA responses in the mucosal secretions were similar between the groups immunized with gD or

with gD in liposomes. These results indicate that induction of systemic (serum) responses was enhanced by liposome delivery, whereas induction of local antibody response was not. One explanation for these results is that the gD molecule is capable of binding to cells (it is a viral protein involved in cell adhesion) and therefore it is likely that gD could by itself bind to epithelial cells.

Milestone 5. Screening other antigens with best adjuvant in mice.

Current work in our laboratory is been designed to evaluate other vaccine antigens using liposomes as adjuvants. This experiments include characterization of liposome formulation using *Haemophilus somnus* antigens (Tbp1, Tbp2, Lpp and HSP). We will be testing the ability of these formulations to induce mucosal and systemic immune responses in mice.

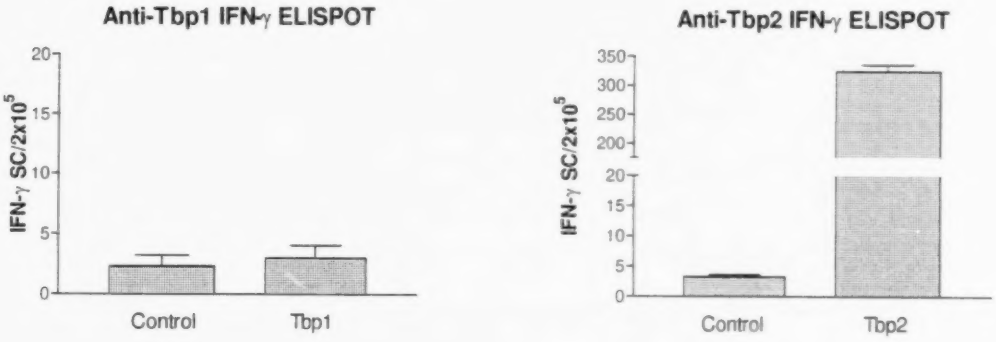


Fig. 11. Interferon gamma secreting cells (IFN-γ SC) in mice immunized with Tbp1 and Tbp2. Mice were immunized with 20 μg of Tbp1 or Tbp2 and boosted three weeks later. Spleen cells were isolated one week after the boost and cultured in the presence of either Tbp1 or Tbp2.

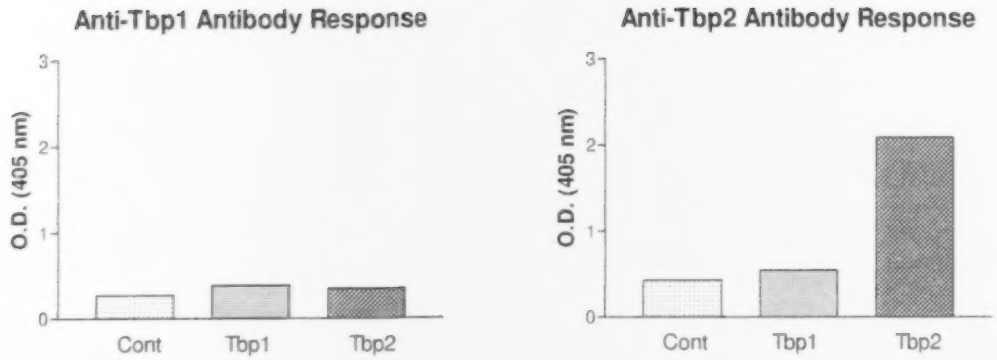


Fig. 12. Antibody response in mice immunized with Tbp1 and Tbp2 antigens. Animals were immunized as in Fig. 11. The antibody levels in serum indicate that Immunization with Tbp1 did not induce Tbp1 antibodies. In contrast, immunization with Tbp2 induced good antibody levels in mice.

We have established the immunological assays to evaluate immune responses to Tbp1 and Tbp2 in mice. This battery of assays will allow us to carry out more specific experiments with the aim of evaluating the efficacy of different adjuvants and delivery systems using this vaccine antigens. All this preliminary work is important before we can take this vaccine into cattle.

This preliminary results showed that Tbp1 does not induce cellular (IFN-γ) or humoral (antibody) responses. In contrast, Tbp2-immunized mice showed high levels of both cellular and humoral responses. More experiments are needed to explore the mechanisms responsible for this discrepancy.

Milestone 7. Injection site reaction study in swine and calves.

Milestone 8. Residue at injection site in swine and calves.

Vaccines with mineral oil adjuvants that produce extensive tissue irritation or leave a residue that reduces meat quality are not acceptable to the pork industry. Swine were used to evaluate the local effects of subunit vaccines prepared from extracts of *Actinobacillus pleuropneumoniae* serotype 1 (ANEX) containing wide variety of adjuvants. These experiments were designed to evaluate tissue damage and protection against *A. pleuroneumoniae* infection.

Animals were vaccinated by an intramuscular injection of different vaccine formulations containing different adjuvants. Each vaccine was injected in the same location in each pig. All pigs were given all injections on day 0, and one or more pigs were euthanized and the injection site examined on different days following infection (see Table 4 and 5).

Table 4. Lesion score[1] for pigs at postmortem.

Adjuvant	Days after vaccination								
	1	3	7	14	21	21	21	28	28
PEG	1	0	0	1	0	0	0	0	0
Lipposyn II	1	1	1	0	1	0	0	0	0
Canola oil	1	1	1	0	0	0	0	0	0
Sesame oil	1	1	1	1	0	2	0	0	0
Corn oil	1	1	2	1	2	0	2	0	1
Peanut oil	1	1	2	1	2	2	2	1	3
Marcol-52 (mineral oil)	1	1	2	3	2	0	3	2	2
QuilA	3	3	3	3	2	3	0	0	0

A lesion score of 0 indicates normality; 1 indicates discoloration of fascial thickening; 2 indicates discoloration and firm thickening; and 3 indicates a multifocal nodular lesion or abscess.

Since pigs are often immunized more than once, we determined whether a secondary immunization could result in exacerbation of tissue damage.

Table 5. Lesion score for pigs at postmortem after a second vaccination with various adjuvants. The scoring system is the same as described for Table 4.

Adjuvant	% of oil in vaccine	Days after vaccination				
		1	3	7	14	21
PEG	none	0	1	0	0	0
Lipposyn II	5%	1	0	0	0	0

Amphigen	5%	0	0	1	0	0
Canola oil	50%	1	2	2	1	1
QuilA	none	1	2	2	3	3
Marcol-52 (second)	5%	1	2	2	2	1
Marcol-52 (second)	50%	0	2	3	2	3
Marcol-52 (first)	50%	3	3	3	3	3

Marcol-52 mineral oil adjuvant resulted in the greatest degree of tissue damage and was characterized by focal linear streaking indicating tissue changes that are not acceptable in a food-producing animal.

Table 6. Vaccine and challenged trial #1.

Group	Adjuvant	Antibody titre[1]	Tissue score[2]	Body temp[3]	Clinical score[4]	Mortality[5]
1	Marcol-52	63800	ND	40.0±0.5	0.25	0/8
2	Emulsigen-Plus	8600	0	40.2±0.7	0.375	2/8
3	QuilA	6100	0	40.4±0.6	1.125	3/8
4	Alhydrogel	1000	0	40.6±0.7	0.875	4/8
5	Avridine	4200	0	40.4±0.9	1.25	6/8
6	Marcol-52 (no antigen)	100	0	39.7	1.5	7/8

Group	Adjuvant	Antibody titre[1]	Tissue score[2]	Body temp[3]	Clinical score[4]	Mortality[5]
1	Canola and vitamin E	1900	3.0	40.6±0.5	0.5	0/8
2	Canola	400	0	41.1±0.2	1.0	4/8
3	Amphygen and vitamin E	3200	4.5	40.0±0.3	0.4	1/6

4	Amphigen	10800	0	40.2±0.4	0.1	0/8
5	Vitamin E (no antigen)	100	0	41.4±0.2	1.5	7/8
6	Vitamin E	2700	2.5	40.1±0.2	0.6	3/8

[1] Geometric mean antibody titer. With each column, numbers with different letter superscripts are significantly different ($p<0.05$).

[2] Sum of the lession score for the 2 injection sites per pig. Scoring system described in Table 4. ND = not determined.

[3] Arithmetic mean and standard deviation of body temperature in C on the second day of challenge.

[4] Median clinical score for ail survivors during the 3 days after challenge. A clinical score of 0 = normality; 1 = increase respiratory rate, alert, active; 2 = increase respiratory rate labored breathing, depressed, incative; and 3 = moribund severely dyspenic, cyanotic.

[5] Number of pigs that died or were euthanized in extremis over the total in the group.

Among the adjuvants that were tested, mineral oils induced protective immunity, although the mineral oil Marcol-52 resulted in severe tissue reactions. The vegetable oils induced little protective immunity, and some of them were quite irritating. The response to the other materials ranged from little irritation or protection induced be the vaccine containing aluminum hydroxide to effective protection without irritation after vaccination with Amphigen or Emulsigen-Plus combination. In conclusion, swine were protected against disease by a subunit vaccine that did not create unacceptable tissue reaction at the immunization site.

We also assess the effect of different adjuvants in inducing tissue reaction in cattle. The adjuvants tested in this experiment were: Avridine, Emulsigen, mineral oil, almond oil/DDA, VSA and. These adjuvants were injected in different sites and tissue biopsies were taken from the injection site to study the type of cells and the degree of inflammation induce by each adjuvant. The results of this study indicated that when different adjuvants are used at their therapeutic concentration, there is no real difference in their ability to induce a local inflammatory reaction or infiltration of immune cells. We concluded that experimental adjuvants such as VSA, were not less reactive than those already licensed and we proceed to license VSA for use in veterinary vaccines.

However, this data also indicated that there is a need for continuing developing strategies to improve vaccine adjuvants and immunization methods that will be of benefit to the livestock industry.

Testing of immune responses to other antigens in swine and calves.

We are currently assessing the immunogenicity and formulation of other bacterial and viral proteins in cattle and swine. These antigens include *Haemophilus somnus* antigens (Tbp1, Tbp2, Lpp and HSP) in cattle and *Streptococcus suis* antigens such as streptococcal acidic glycoprotein (SAGP) and other putative protective streptococcal antigens in pigs.

Final Conclusions:

The data presented in this report highlights the major results for each milestone outlined in the original application. We have demonstrated that the adjuvant develop in VIDO (VSA), a mineral oil emulsion, is a potent stimulator of immunity and when use in vaccine formulation with gD, it induces protective responses to BHV-1 infection in cattle. However, mineral oil can induce long-term irritation in the animal and mineral oil adjuvants are banned in the European community. Therefore development of novel vaccine adjuvants to replace mineral oil is an important area of research for the Vaccine Formulation and Delivery Team at VIDO. It is for this reason that we focused much of our own research into assessing a more suitable, biodegradable oil such as liposomes or cytokine molecules as potential adjuvants. Our results clearly showed that we are on the right track.

Most infectious diseases involve colonization or invasion of the host through mucosal surfaces. Induction of mucosal immunity as a first line of defense against most pathogens is crucial. However, since it is well recognized that systemic immunization is not always efficient in inducing mucosal immunity, immunostimulation of mucosal sites is best achieved by administration of antigens at mucosal surfaces. Liposomes have not only been proven effective in

delivering vaccine antigens by the systemic route but are also capable to inducing mucosal immunity when orally or intranasally administered. We have started to evaluate whether our liposome vaccine preparations could stimulate development of immunity following systemic and mucosal (intranasal or oral) immunization. Our results in mice suggest that intranasal immunization with gD in liposomes is more efficient in inducing mucosal responses than the vaccine antigen alone. We are now in the process of taking this approach to immunized cattle.

In summary, we have successfully completed many of the milestones outlined in the original application and continue to develop strategies to improve vaccine adjuvants and immunization methods that will be of benefit to the livestock industry.

D) INDICATIONS FOR FURTHER DEVELOPMENT

As indicated in the previous section, we have shown that delivery and route of administration of vaccines influence the immune response they induced. Future studies should focus in the development of delivery methods that will make this technology practical for field applications. In addition, development of alternative methods of vaccine delivery, such as transdermal (through the skin) should be explored.

E) INFORMATION OF BENEFIT TO OTHER PRODUCERS

The potential of developing cost effective vaccine delivery systems such as liposomes provides an opportunity to enhance disease control and safety over currently available vaccines. Preventing disease with better vaccines should improve farm efficiency and profits as well as resulting in consumer savings. Added benefits may be incurred through the reduction of carcass trim and hide

damage due to reactive vaccines and excessive antibiotic treatment. VIDO continues to update producers groups on the results of this research on an ongoing basis.

PERSONNEL

The following people contributed to this project: Dr. Richard Harland, Ms. Marlene Snider, Dr. Oksana Yarosh, Ms. Gillian Merrifield, Ms. Jane Fitzpatrick, Ms. Catherine Ewen, Dr. Qifu Xiao, Dr. Phil Willson, Dr. Joyce Van Donkersgoed and Dr. Maria Baca-Estrada. Some of these individuals' salaries were paid by this grant. Please refer to the financial statement for salary and time allocation.

EQUIPMENT

There was no equipment purchased with this grant.

PROJECT DEVELOPMENT MATERIALS

Copies of VIDO's Annual Reports of the last 3 years are included with this report.

SDAF ACKNOWLEDGMENT

VIDO's Annual Report acknowledged SDAF support. Acknowledgment to SDAF can also be found in the publications enclosed.

EXPENSE STATMENT

A final expense report will be sent in the middle of July 1997. The reason for this is the time it takes the University Accounting Department to process the financial statements for individual accounts.

VIRAL IMMUNOLOGY
Volume 9, Number 1, 1996
Mary Ann Liebert, Inc.
Pp. 11–22

Immunogenicity of Bovine Herpesvirus 1 Glycoprotein D in Mice: Effect of Antigen Form on the Induction of Cellular and Humoral Immune Responses

M.E. BACA-ESTRADA, M. SNIDER, S.K. TIKOO, R. HARLAND, L.A. BABIUK, and S. VAN DRUNEN LITTEL-VAN DEN HURK

ABSTRACT

For the development of veterinary subunit vaccines, modifications to the antigen may be needed to make the production of these vaccines cost effective. To investigate the effect of antigen modifications on immune response, we used glycoprotein D, one of the major glycoproteins of bovine herpesvirus-1 (BHV-1), as a model antigen. We developed a mouse model to assess the immune response elicited by immunization with either a recombinant truncated (tgD) or the authentic full-length (gD) form of BHV-1 gD in VSA3, a novel water-in-oil adjuvant. Both forms of BHV-1 gD antigen induced good levels of cell-mediated immunity, as evaluated by antigen-specific proliferative response and cytokine (IFN-γ and IL-4) production. Following primary immunization, the humoral immune response induced by gD was superior to that elicited by vaccination with tgD. However, after a secondary immunization, a strong and similar antibody response to BHV-1 gD was induced by both forms of the antigen. The difference in immunogenicity between gD and tgD after primary immunization was not due to the loss of immunogenic epitopes in the truncated antigen or the ability to associate with the adjuvant VSA3. Our results indicate that both gD and tgD are capable of efficiently inducing a cell-mediated immune response, and although recombinant tgD is less efficient in inducing a primary humoral immune response when compared to the full-length gD, tgD effectively primed for a secondary antibody response.

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) is an important pathogen of cattle associated primarily with two significant economic diseases, infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (9,32). In addition, BHV-1 infections may lead to fatal pneumonia in association with secondary bacterial infections, a respiratory disease syndrome called "shipping fever," which causes heavy economic losses to the cattle industry (32). Immunization of cattle with current modified live and killed BHV-1 vaccines appears to induce some level of protection against the disease (7,8,16,20,25), but they are not always effective (14,15). Furthermore, live attenuated vaccines have been associated with the induction of abortions, adverse

Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E3.

reactions including immunosuppression (10,14,19), and reactivation of the vaccine strain (23). Thus, there is a great need to develop vaccines to not only prevent infection and latency, but also to prevent virus spread and induction of immunosuppression. In addition, for eradication purposes, it is important to be able to differentiate vaccinated from carrier animals.

Effective protection of cattle against challenge with BHV-1 and *Pasteurella haemolytica* has been achieved by immunization with a subunit vaccine consisting of BHV-1 glycoprotein D (gD) (29). Although immunization with other BHV-1 glycoproteins such as gB and gC individually or in combination, conferred protection from disease, gD was found to be more efficient in reducing viral replication, shedding, and clinical signs (1,28). An additional feature of gD is its ability to induce a strong cellular immune response as compared to that induced by gB or gC (13). Based on this information, the gD antigen was selected for the development of a BHV-1 subunit vaccine.

For the manufacture of veterinary subunit vaccines, modifications to the antigen are needed to make the production of these vaccines cost effective. Recently, an economical method to produce gD has been developed by engineering the gD gene to function under the control of a bovine heat-shock protein 70 gene promoter, which regulates the expression of gD in transfected MDBK cells (17). This system was designed to produce a truncated form of gD (tgD), which was generated by removing the transmembrane domain (27). Large quantities of tgD were produced in the transfected MDBK cells and efficiently secreted into the medium. The tgD produced in this system was antigenically similar to the authentic molecule when analyzed *in vitro* against a panel of gD-specific monoclonal antibodies (17). Furthermore, recent studies in cattle demonstrated that immunization with tgD conferred complete protection from BHV-1 infection (30).

Proper folding, oligomerization and glycosylation have been shown to be important factors in maintaining immunogenicity and efficacy of recombinant antigens (4,22,26). Furthermore, antigen modifications can change the type of immune response induced, leading to the stimulation of differential patterns of cytokine synthesis (31). In this study, we have established a mouse model to further characterize the type of immune responses elicited by gD and to assess whether the removal of the transmembrane domain influences these responses.

MATERIALS AND METHODS

Mice/immunizations. Five to six-week-old C57BL/6 mice were purchased from the Charles River facility in Kingston, Ontario, Canada. Groups of 5 mice were vaccinated subcutaneously with different amounts of recombinant tgD or gD, prepared as previously described (30), and formulated with VSA3 (Biostar, Saskatoon, Saskatchewan, Canada). In some experiments animals were reimmunized on day 21. Control groups received VSA3 without antigen. Animals were bled following primary or secondary immunization by cardiac puncture, under halothane anaesthesia.

Cell preparation. Spleens and lymph nodes were aseptically removed from naive and immune mice and teased through a nylon mesh. Most of the red cells were removed in a 1 min lysis step using Tris buffered ammonium chloride (0.75%). They were washed twice and subsequently resuspended in culture medium.

Culture medium consisted of RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co.), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma Chemical Co.), 2 mM L-glutamine (Gibco, Life Technologies, Burlington, Canada), 100 μM nonessential amino acids (Gibco, Life Technologies), 1 mM sodium pyruvate (Gibco, Life Technologies), 10 mM HEPES (Gibco, Life Technologies), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.).

Proliferation. Two $\times 10^5$ spleen cells were dispensed in 100 μl volumes into the wells of microtitre plates. UV irradiated BHV-1 (5×10^5 PFU/ml) and various concentrations of gD antigen were added in a 100 μl volume to triplicate wells. After 3 days in culture the cells were labeled with [^{3}H]thymidine (Amersham, Oakville, Canada) at a concentration of 0.4 μCi/well. The cells were harvested 18 h later and thymidine incorporation was assessed by scintillation counting. Proliferative responses were expressed as a stimulation index (counts per minute in the presence of antigen/counts per minute in the absence of antigen).

ELISPOT. Cytokine-specific ELISPOT assay was used as previously described (6). Briefly, spleen cells were incubated in culture medium at 37°C and 5% CO_2 for 24–48 h in the presence or absence of gD (0.1

μg/ml). The cells were washed twice and resuspended to the appropriate concentration in culture medium. Nitrocellulose plates (Millipore Multiscreen-HA, Millipore, Bedford, MA) were coated for 2 h at ambient temperature with 2 μg/ml of purified antimouse interleukin (IL)-4 (11B11) or 5 μg/ml of purified antimouse interferon (IFN)-γ (R4-6A2) (Pharmingen, San Diego, CA) diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6). Unbound antibody was removed by washing once in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma Chemical Co.) (PBS-T) and three times with PBS. This was followed by a blocking step in culture medium for 2 h. The medium was decanted and 100 μl of each cell suspension was added to triplicate wells. After an overnight incubation, the plates were washed in cold PBS-T to remove all cells. Biotinylated antibodies specific to mouse IL-4 (BVD6-24G2) and IFN-γ (XMG1.2) (Pharmingen) were diluted to a concentration of 3 μg/ml in 0.1% BSA/PBS. One hundred microliters of each suspension was added to the respective wells and incubated for 2–4 h at ambient temperature. The plates were washed three times in PBS-T. A 1/1000 dilution of streptavidin-alkaline phosphatase (BIO/CAN Scientific, Mississauga, Ontario, Canada) in 0.1% BSA/PBS was prepared and dispensed in 100 μl volumes. Incubation was for 2 h at ambient temperature followed by 8 consecutive washes in PBS. The substrate was prepared as follows: 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.) was dissolved in dimethylformamide (Sigma Chemical Co.) to a 15 mg/ml concentration and 1.5 mg was added to 10 ml of a 15 mM borate buffer (pH 9.8) containing 3 mg of nitroblue tetrazolium (NBT) (Sigma Chemical Co.). Magnesium chloride was added to a 5 mM concentration. The substrate was filtered and added to the wells in 100 μl volumes and incubated at room temperature for 10–60 min. The plates were washed in distilled water and subsequently air dried. Spots were counted using a dissecting microscope. Values are expressed as the number of positive, stained spots per 10^5 cells.

ELISA. Antibody titers specific for gD were determined by ELISA. Microtiter plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, VA) were coated with purified gD or tgD at a concentration of 0.05 μg/well. The dilution was made in a carbonate/bicarbonate buffer (pH 9.6). The plates were incubated for 2 h at 37°C and then washed 4 times in PBS-T. Four-fold dilutions of mouse sera were prepared in PBS-T and dispensed in 200 μl volumes. A 2 h incubation and wash step followed. Affinity-purified horseradish peroxidase-conjugated goat antimouse Ig (Boehringer Mannheim, Laval, Quebec Canada), at a dilution of 1/10,000, was used as the detecting antibody. After incubation for 2 h and four subsequent washes, 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS) (Boehringer Mannheim) was used to develop the assay. The absorbance was read after 10 min at 405 nm with a reference wavelength of 490 nm.

Serum neutralization. Sera from immunized mice were analyzed by a BHV-1 plaque reduction assay as described previously (2). The titers were expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control

Statistical analyses. Data were entered into a database in the statistical analysis program Instat Graph Pad™. Antibody titers were analyzed using the $\log_2$ of the reciprocal of the dilution and the results for groups were expressed as geometric means. Differences in antibody titers and stimulation indices among vaccine groups were investigated using the nonparametric Mann–Whitney U test. The difference between *in vitro* stimulation conditions was calculated using a paired Student's t test.

RESULTS

Humoral immune response to full-length and truncated gD in mice. To evaluate whether the immunogenicity of recombinant tgD differed from that of the full-length molecule, groups of 5 mice were immunized with different amounts of tgD or gD, as indicated in Figure 1. The immune response to gD was only analyzed using 1 and 0.1 μg, since previous studies demonstrated these to be the optimal doses required to stimulate proliferative responses to gD in mice (O. Yarosh et al., personal communication). Analysis of sera by ELISA showed that after one immunization, tgD was able to induce good levels of specific antibodies in a dose-dependent manner (Fig. 1A). Similarly, the sera from these animals contained good levels of BHV-1 neutralizing antibodies (Fig. 1B). However, comparison of the antibody levels induced by the two different forms of gD indicated that immunization with 0.1 or 1 μg of gD induced significantly higher ELISA ($p < 0.05$) and BHV-1 neutralizing antibody ($p < 0.05$) titers than those induced by the same amount

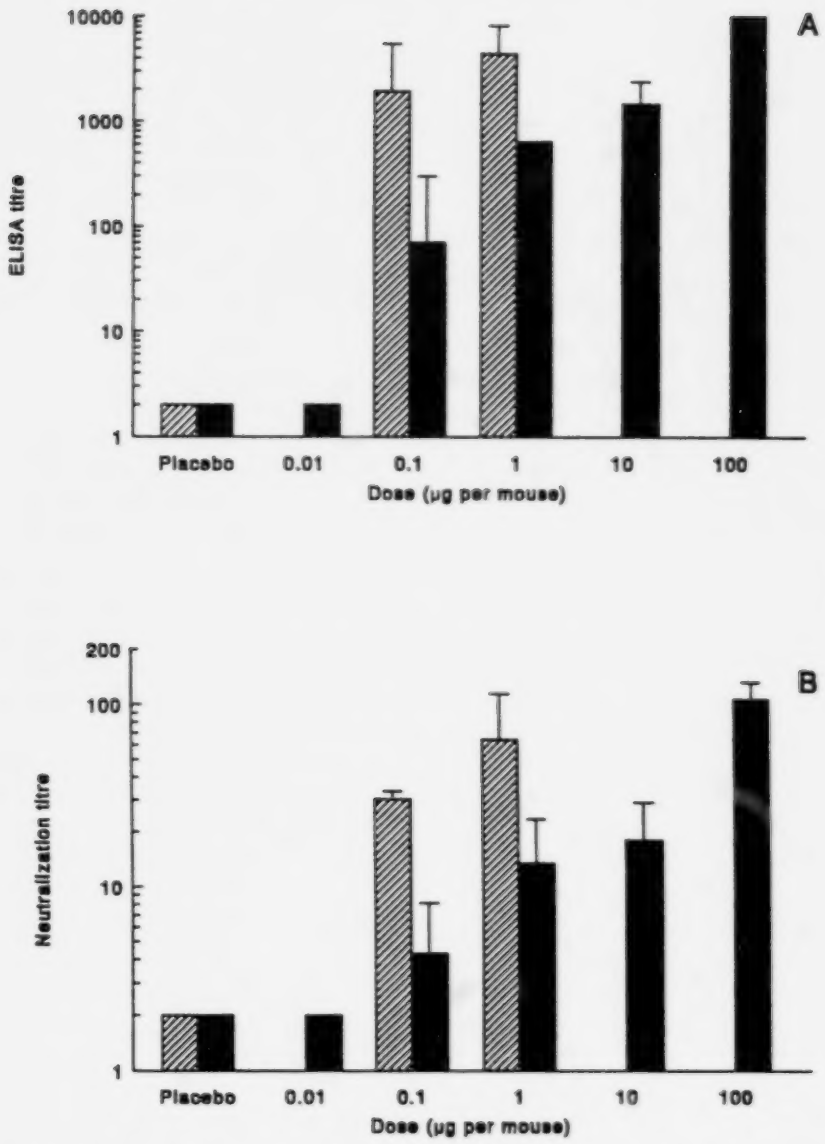


FIG. 1. Serum antibody responses following a single immunization with different doses of gD (hatched) and tgD (solid). (A) gD-specific ELISA titers expressed as the reciprocal of the highest dilution resulting in a reading of three standard deviations above the control value. (B) BHV-1 neutralizing antibody titers expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control. Results are expressed as geometric means and bars indicate the standard deviation of 5 mice.

of tgD (Fig. 1). Following a booster immunization, the serum ELISA and BHV-1 neutralization antibody titers increased in all groups (Fig. 2). Interestingly, after secondary immunization, no statistical differences were observed between the gD-specific antibody levels induced by the two different forms of the gD antigen, except for the virus neutralizing titers of animals immunized with 0.1 μg (Fig. 2). None of the control VSA3-immunized animals produced any specific immune response.

Cellular immune response to full-length and truncated gD in mice. To assess the ability of gD and tgD in inducing cell-mediated immune responses, spleen cells from tgD or gD-immunized mice were stimulated *in vitro* with gD or BHV-1. Antigen-specific responses were measured by cellular proliferation and lymphokine secretion. In contrast to the dose-dependent antibody response induced by tgD, the optimal im-

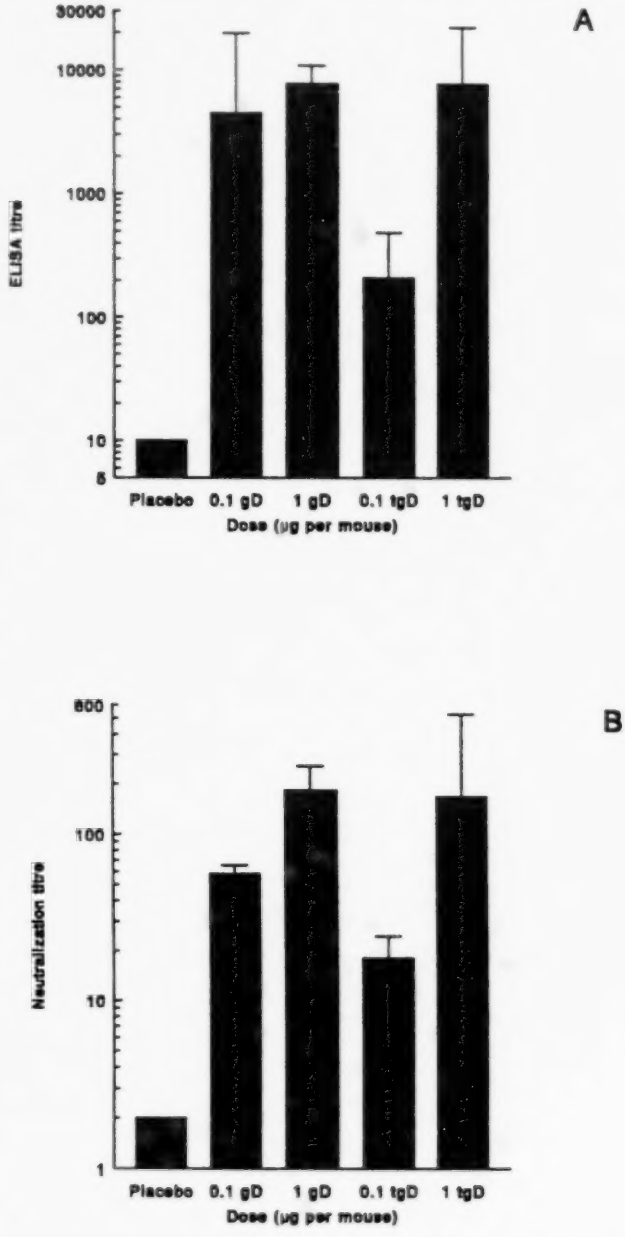


FIG. 2. Secondary antibody responses in serum following two immunizations with gD and tgD at a 2 week interval. (A) gD-specific ELISA titers expressed as the reciprocal of the highest dilution resulting in a reading of three standard deviations above the control value. (B) BHV-1 neutralizing antibody titers expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control. Results are expressed as geometric means and bars indicate standard deviation of 5 mice.

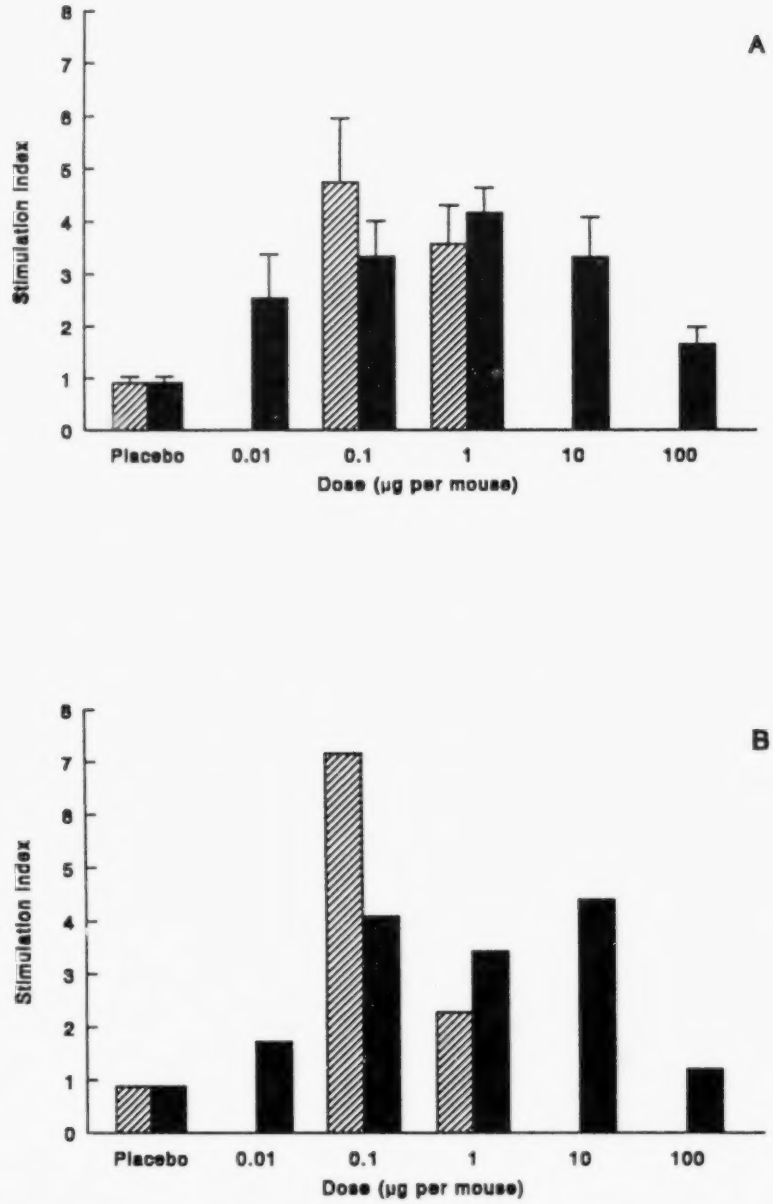


FIG. 3. Proliferative responses of (A) spleen cells and (B) draining lymph nodes following a single immunization with different doses of gD (hatched) and tgD (solid). Results are expressed in (A) as mean stimulation index and bars indicate standard deviation of 5 mice and in (B) as stimulation index of pooled lymph nodes from 5 mice.

munizing dose for induction of gD-specific proliferative responses was between 0.1 and 10 μg. Vaccination with 100 μg/mouse induced poor proliferative responses in spleen (Fig. 3A) and draining lymph node cells (Fig. 3B). After secondary immunization, both gD and tgD antigens induced consistently good antigen-specific cell-mediated responses as demonstrated by cell proliferation against purified gD protein (Fig. 4A) or UV-irradiated BHV-1 virus (Fig. 4B). In contrast to the humoral immune response, the antigen-specific proliferative response was higher in the tgD-immunized mice.

To further examine the cell-mediated immune responses induced by the different forms of gD, we assessed the cytokine profile elicited in the spleen of immunized animals. Following *in vitro* restimulation with gD, we determined the number of IFN-γ and IL-4-secreting cells (Fig. 5). Glycoprotein D and tgD induced similar levels of IFN-γ and IL-4-secreting cells after secondary immunization (Fig. 5). In addition, high levels of IL-4-secreting cells were observed in control VSA3-immunized mice (Fig. 5).

Truncation of gD affects antigen presentation but not immunogenic epitopes. To assess whether the removal of the hydrophobic transmembrane domain caused changes that affected antigen presentation, we determined the ability of gD and tgD to mediate *in vitro* restimulation of primed spleen cells in an antigen-specific cell proliferation assay. Spleen cells from gD or tgD-immunized mice were cultured in the presence of 0.1 μg/ml of gD or tgD. Significantly lower responses were observed when tgD was used as an antigen for *in vitro* stimulation of both gD- and tgD-primed spleen cells (Fig. 6).

Previous studies have shown that tgD was antigenically similar to the authentic gD when analyzed *in vitro* against a panel of gD-specific monoclonal antibodies (17). To further explore whether immunization with tgD influences the specificity of the antibodies, the serum antibody titers of gD- and tgD-immunized animals were measured by ELISA on gD- and tgD-coated plates. Since the antibody titers determined against gD and tgD were very similar, this confirms that gD and tgD have the same antigenic epitopes (Fig. 7). Since most of the relevant, neutralizing epitopes on gD are conformation dependent (12,26), the truncated form of gD is very likely posttranslationally processed and configured similar to the full-length molecule.

DISCUSSION

In this study we have evaluated the immunogenicity of BHV-1 tgD, a recombinant glycoprotein that lacks the transmembrane anchor domain (17,27), and compared it to the immune response induced by the authentic full-length gD molecule. Following primary immunization, the humoral immune response induced by gD was superior to that elicited by vaccination with tgD. However, both antigens were equally capable of inducing cell-mediated immune responses. Interestingly, immunization with tgD efficiently primed ani-

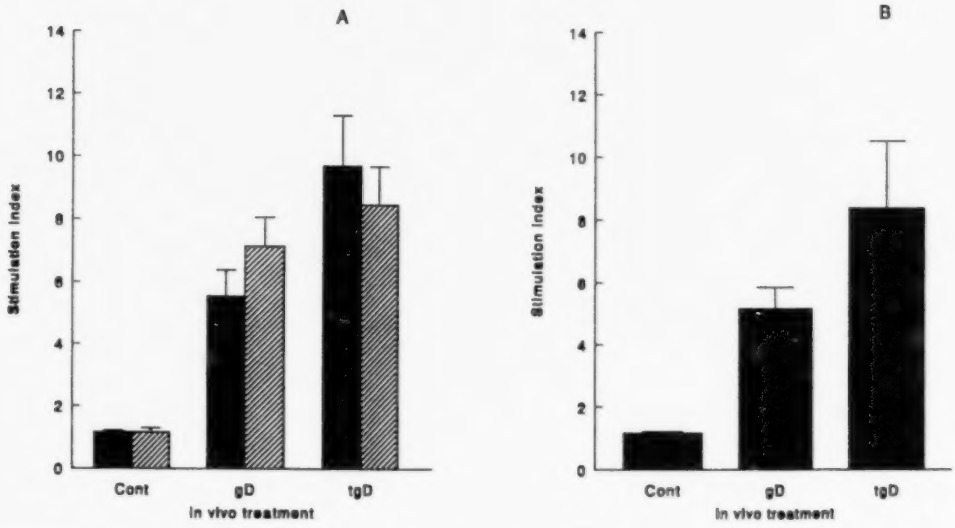


FIG. 4. Proliferative responses of spleen cells following *in vitro* stimulation with (A) gD, 0.01 μg/ml (solid) or 0.1 μg/ml (hatched) and (B) UV-irradiated BHV-1 equivalent to 5×10^5 pfu/ml before irradiation. Spleen cells were obtained from mice immunized with 0.1 μg of gD or tgD, vaccination was repeated on day 21, and the animals were sacrificed 10 days later. Results are expressed as mean stimulation index and the bars indicate the standard deviation of three independent experiments.

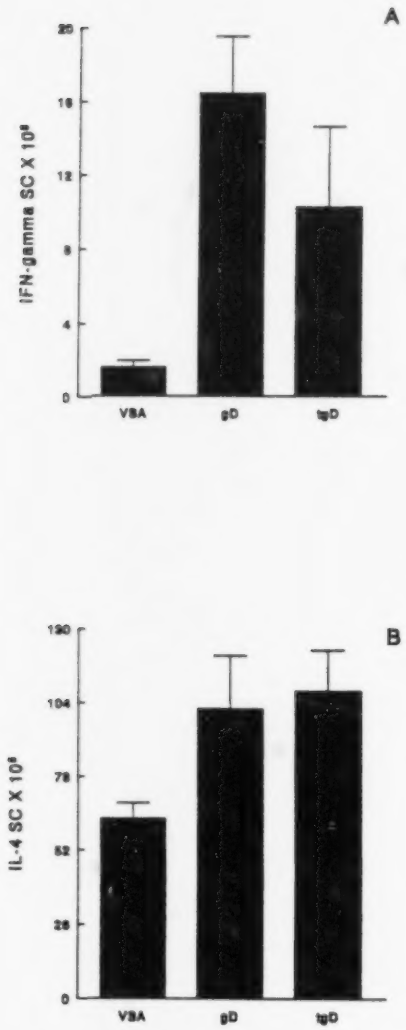


FIG. 5. Number of cytokine-secreting cells following *in vitro* stimulation of spleen cells with gD. Mice were immunized and boosted 2 weeks later. Spleen cells were harvested 10 days after the second immunization and cultured in the presence of purified gD for 48 h prior to detection of cytokine. Results are expressed as the mean plus standard deviation of (A) IFN-γ or (B) IL-4-secreting cells/10^6 cells of triplicate wells of pooled spleen cells from 5 mice.

mals for a strong gD-specific humoral immune response following a secondary immunization, which was equivalent to the response to gD.

Although gD appeared superior to tgD in inducing primary humoral immune responses, this difference in immunogenicity is unlikely to be due to the loss of immunogenic epitopes in the truncated antigen, as shown by the ability of sera from gD-immunized mice to bind equally to both tgD- and gD-coated plates. In addition, no differences were observed in the ability of gD and tgD to associate with VSA3 (data not shown), indicating that our findings are not related to changes in the capacity of antigen to associate with the adjuvant.

Thus, the difference in immunogenicity may be related to the fact that truncation of gD alters the ability of the glycoprotein to form oligomers (van Drunen Littel-van den Hurk et al., submitted). Therefore, the reduced immunogenicity observed with tgD is not related to the loss of immunogenic epitopes but rather

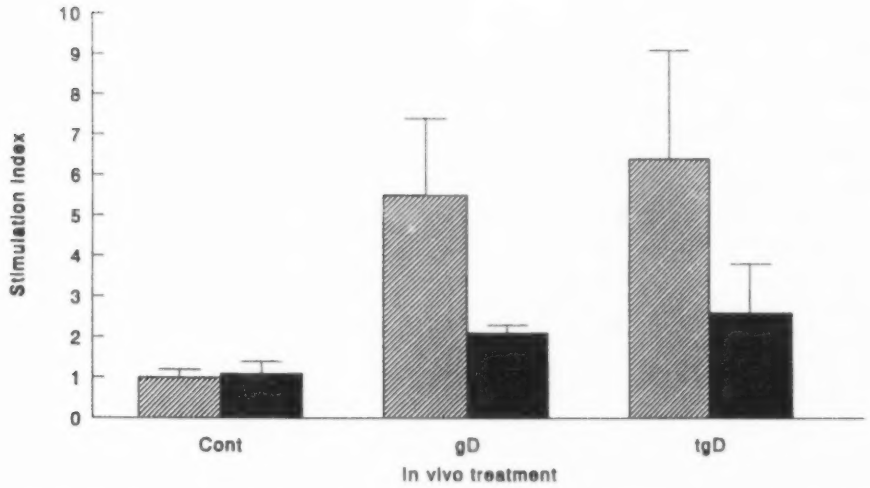


FIG. 6. Comparison between gD and tgD to mediate *in vitro* proliferative responses. Groups of five mice were immunized with 0.1 μg of gD or tgD. Proliferative responses on day 21 were determined by culturing the spleen cells in the presence of 0.01 μg/ml of gD (hatched) or tgD (solid). Results represent the mean and standard deviation of three independent experiments.

to changes in the molecule resulting from the removal of the hydrophobic transmembrane domain, which reduce the formation of oligomers and subsequently the immunogenicity of the antigen. Indeed, aggregation of soluble proteins by structural alterations introduced by lipid conjugation (11) or by physical combination with liposomes (21) or adjuvants (5) has been shown to enhance immunogenicity of proteins, both *in vitro* and *in vivo*. These data support our findings that tgD is less efficient in mediating *in vitro* restimulation of primed spleen cells. One possible mechanism may be that complexed antigen may be presented better by antigen-presenting cells, than soluble molecules. Vaccination of mice with tgD induced a humoral immune response that was directly proportional to the amount of immunizing antigen. In contrast, spleen

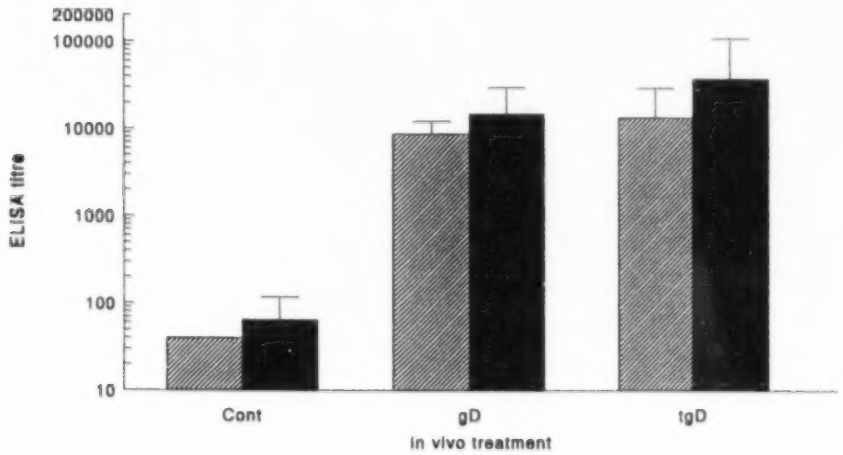


FIG. 7. Comparison between gD (hatched) and tgD (solid)-specific ELISA to measure serum antibody levels induced by immunization with gD or tgD. Groups of five mice were immunized twice at a 2 week interval with 1 μg of gD or tgD. Results represent the mean and standard deviation and ELISA titers were expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value.

cells from mice given the highest immunizing dose of tgD showed the lowest *in vitro* proliferative responses. These results are consistent with other studies using herpes simplex virus gD as a subunit vaccine in rhesus monkeys (18), where proliferative responses were inversely proportional to the amount of antigen administered. Moreover, it has been shown that high doses of antigen preferentially induce antibody responses rather than cell-mediated immunity. Thus, the dose of antigen given *in vivo* is crucial in determining the class of immune response induced (3,24), therefore, it is possible that a similar mechanism is responsible for the preferential induction of an antibody response in mice immunized with the higher doses of tgD.

Immunization with gD or tgD formulated in VSA3 elicited IFN-γ and IL-4-secreting cells after *in vitro* restimulation with gD. These observations suggest that the nature of T cells developed after immunization with this adjuvant formulation include cells from the two major subsets: Th1 and Th2. However, control animals that received VSA3 alone showed elevated number of IL-4-producing cells. This nonspecific stimulation of IL-4 by the vaccine formulation was observed only within the first week after immunization (Fig. 5), suggesting that the adjuvant is capable of nonspecifically stimulating the production of IL-4. Therefore, this immunomodulatory effect may be an important element in the adjuvanticity of VSA3.

Collectively, our findings support the observations that both gD and tgD are highly immunogenic antigens capable of inducing humoral and cellular immune responses. Although differences in the levels of humoral immune responses were observed between the full-length and the truncated antigens, these did not appear to be related to the loss of any immunogenic epitopes or changes in the antigen–adjuvant association, but rather to the primarily monomeric nature of the truncated glycoprotein. This model will allow us not only to assess improvements in immunogenicity but also to evaluate new strategies to selectively induce particular immune responses, including antigen-specific CTL responses.

ACKNOWLEDGMENTS

The authors thank Brenda Karvonen for her excellent technical assistance, and the animal support staff at VIDO for care and handling of the animals. This work was supported by grants from the National Science and Engineering Council of Canada, the Medical Research Council of Canada, Agriculture Development Fund of Saskatchewan, and the Alberta Agricultural Research Institute. M. E. B-E. was a Fellow of the Medical Research Council of Canada. Published with permission of the Director as Journal Series No. 202.

REFERENCES

1. Babiuk, L.A., J. L'Italien, S. van Drunen Littel-van den Hurk, T.J. Zamb, M.J.P. Lawman, G. Hughes, and G.A. Gifford. 1987. Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual glycoproteins. Virology 159:57–66.
2. Babiuk, L.A., R.C. Wardley, and B.T. Rouse. 1975. Defense mechanisms against bovine herpesvirus: Relationship of virus host cell events to susceptibility to antibody-complement lysis. Infect. Immun. 12:958–963.
3. Bretscher, P.A., G. Wei, J.N. Menon, and H. Bielefeldt-Ohmann. 1992. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Lieshmania major*. Science 257:539–542.
4. Collins, P.L., and G. Mottet. 1991. Homooligomerization of the hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3 occurs before the acquisition of correct intramolecular disulfide bonds and mature immunoreactivity. J. Virol. 65:2362–2371.
5. Cox, J.C., and A.R. Coulter. 1992. Advances in adjuvant technology and application. Pp. 49–111. In W.K. Yong (ed.): Animal parasite control utilizing biotechnology. CRC Press, Boca Raton, FL.
6. Czerkinsky, C., G. Andersson, H.P. Ekre, L.A. Nilsson, L. Klareskog, and O. Ouchterlony. 1988. Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J. Immunol. Methods 110:29–36.
7. Frerichs, G.N., S.B. Woods, M.H. Lucas, and J.J. Sands. 1982. Safety and efficacy of live and inactivated infectious rhinotracheitis vaccines. Vet. Rec. 111:116–122.

8. Gerber, J.D., A.E. Marron, and C.J. Kucera. 1978. Local and systemic cellular and antibody immune responses of cattle to infectious bovine rhinotracheitis virus vaccine administrated intranasally or intramuscularly. Am. J. Vet. Res. 39:753–760.

9. Gibbs, E.P.J., and M.M. Rweyemamu. 1977. Bovine herpesvirus I. Bovine herpesvirus-1. Vet. Bull. (London) 47:317–343.

10. Harland, J.R., A.A. Potter, S. van Drunen-Littel-van den Hurk, J. Van Donkersgoed, M.D. Parker, T.J. Zamb, and E.D. Janzen. 1992. The effect of subunit or modified live bovine herpesvirus-1 vaccines on the efficacy of recombinant *Pasteurella haemolytica* vaccine for the prevention of respiratory disease in feedlot calves. Can. Vet. J. 33:734–741.

11. Hopp, T.P. 1984. Immunogenicity of a synthetic HBsAg peptide enhancement by conjugation to fatty acid carrier. Mol. Immunol. 21:13–16.

12. Hughes, G., L.A. Babiuk, and S. van Drunen Littel-van den Hurk. 1988. Functional and topographical analyses of epitopes on bovine herpesvirus type 1 glycoprotein IV. Arch. Virol. 103:47–60.

13. Hutchings, D.L., S. van Drunen Littel-van den Hurk, and L.A. Babiuk. 1990. Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. J. Virol. 64:5114–5122.

14. Hyland, S.J., B.C. Easterday, and R. Pawlisch. 1974. Antibody levels and immunity to infectious bovine rhinotracheitis virus (IBR) infection in Wisconsin dairy cattle. Dev. Biol. Stand. 28:510–525.

15. Jensen, R., R.E. Pierson, P.M. Braddy, D.S. Saari, L.H. Lauerman, J.J. England, H. Keyvanfar, J.R. Collier, D.P. Horton, A.E. McChesney, A. Benitez, and R.M. Christie. 1976. Shipping fever pneumonia in yearling feedlot cattle. J. Am. Vet. Med. Assoc. 169:500–506.

16. Jericho, K.W.F., and L.A. Babiuk. 1983. The effect of dose, route and virulence of bovine herpesvirus-1 vaccine on experimental respiratory disease in cattle. Can. J. Comp. Med. 47:133–139.

17. Kowalski, J., S.A. Gilbert, S. van Drunen Littel-van den Hurk, J. van den Hurk, L.A. Babiuk, and T.J. Zamb. 1993. Heat-shock promoter-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells. Vaccine 11:1100–1108.

18. Mishkin, E.M., J.R. Fahey, Y. Kino, R.J. Klein, A.S. Abramovitz, and S.J. Mento. 1991. Native herpes simplex virus glycoprotein D vaccine: Immunogenicity and protection in animal models. Vaccine 9:147–153.

19. Mitchell, A. 1974. An outbreak of abortion in a dairy herd following inoculation with an intramuscular infectious bovine rhinotracheitis vaccine. Can. Vet. J. 15:148–150.

20. Nettleton, P.F., and J.M. Sharp. 1980. Infectious bovine rhinotracheitis virus excretion after vaccination. Vet. Rec. 107:379.

21. Oth, D., G. Mercier, P. Perrin, M.L. Joffret, P. Sureau, and L. Thibodeau. 1987. The association of the rabies glycoprotein with liposome (immunosome) induces an *in vitro* specific release of interleukin 2. Cell Immunol. 108:220–226.

22. Parks, G.D., and R.A. Lamb. 1990. Folding and oligomerization properties of a soluble and secreted form of the paramyxovirus hemagglutinin-neuraminidase glycoprotein. Virology 178:498–508.

23. Pastoret, P.P., L.A. Babiuk, V. Misra, and P. Griebel. 1980. Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovine rhinotracheitis and dexamethasone. Infect. Immun. 29:483–488.

24. Salk, J., P.A. Bretscher, P.L. Salk, M. Clerici, and G.M. Shearer. 1992. A strategy for prophylactic vaccination against HIV. Science 260:1270–1272.

25. Thiry, E., B. Brouchier, J. Saliki, M. Pirak, and P.P. Pastoret. 1985. Excretion and reexcretion of thermosensitive and wild-type strains of infectious bovine rhinotracheitis virus after co-infection or two successive infections. Vet. Microbiol. 10:371–380.

26. Tikoo, S.K., M.D. Parker, J.V. van den Hurk, J. Kowalski, T.J. Zamb, and L.A. Babiuk. 1993. Role of N-linked glycans in antigenicity, processing, and cell surface expression of bovine herpesvirus 1 glycoprotein gIV J. Virol. 67:726–733.

27. Tikoo, S.K., T.J. Zamb, and L.A. Babiuk. 1993. Analysis of bovine herpesvirus 1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia viruses. J. Virol. 67:2103–2109.

28. Van Drunen Littel-van den Hurk, S., G.A. Gifford, and L.A. Babiuk. 1990. Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 8:358–368.

29. Van Drunen Littel-van den Hurk, S., M.D. Parker, B. Massie, J.V. van den Hurk, R. Harland, L.A. Babiuk, and T.J. Zamb. 1993. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 11:25–35.

30. Van Drunen Littel-van den Hurk, S., J. Van Donkersgoed, J. Kowalski, J.V. van den Hurk, R. Harland, L.A. Babiuk, and T.J. Zamb. 1994. A subunit gIV vaccine, produced by transfected mammalian cells in culture, induces mucosal immunity against bovine herpesvirus-1 in cattle. Vaccine 12:1295–1302.

31. Yang, X., R.S. Gieni, T.R. Mosmann, and K.T. HayGlass. 1993. Chemically modified antigen preferentially elicits induction of Th1-like cytokine synthesis patterns in vivo. J. Exp. Med. 178:349–353.

32. Yates, W.D.G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46:225–263.

Address reprint requests to:
S. van Drunen Littel-van den Hurk
Veterinary Infectious Disease Organization
120 Veterinary Rd.
University of Saskatchewan
Saskatoon, SK. Canada S7N 5E3